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PATENT SPECIFICATION 934,554

DRAWINGS ATTACHED.

934,554

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COMPLETE SPECIFICATION.

Methods of Obtaining Substantially Stable Concentrated Extracts of Red Vine (Vitis Vinifera) and the Extracts Resulting Therefrom.

I, EMILE ABELES, a French Citizen of 42 Avenue Augustin Dumont, Malakoff, Seine, France, do hereby declare the invention, for which I pray that a patent may be granted to me, and the method by which it is to be performed, to be particularly described in and by the following statement :—

The present invention relates to therapeutic preparations having an important physiological action as modifiers of vascular spasms and of capillary permeability, and to methods for obtaining them.

The virtues of the red vine leaf obtainable from a so-called dyeing variety of Vitis Vinifera L. (Ampilides) have been praised for a long time as a remedy against internal haemorrhages, the difficulties of the menopause, painful phlebitis and in the treatment of haemorrhoids. The red vine is generally used in a decoction obtained by infusion and constitutes an efficacious remedy in so-called popular medicine.

These properties have however only been observed empirically and it has never yet been possible to obtain, from the natural raw material which this red vine constitutes, a suitable product with a high and constant activity in a small volume, and it has been impossible to obtain it by a precise and repeatable process. It is for this reason that the present invention in the first place relates to means allowing concentrated extracts of red vine which are more convenient than the leaf to be obtained, the activity of said extracts being of the same nature as the original infusion and their principal advantage residing in a much more intense activity.

The preparation of fluid or soft extract according to the techniques usually used in the pharmaceutical art, starting from dried leaves, allow preparations only of very mediocre quality to be obtained. In fact it has been found that :—

The extract changes in the course of time. It has a brown colour and forms large quantities of sediments. Its colouring does not correspond to that of the original material.

The physiologically perceptible activity rapidly disappears in the course of time.

In particular the invention allows these drawbacks to be avoided, due to the determination of the necessary and sufficient conditions for obtaining a medicament answering the above-mentioned requirements. According to the invention, there is provided a method for obtaining a substantially stable concentrated extract of red vine (Vitis Vinifera), in which dried and crushed leaves of red vine are extracted by warm aqueous alcohol, and the mixture is acidified after cooling, the filtered liquid then being distilled in vacuo in the presence of an antioxidant. The invention also provides substantially stable concentrated extracts of red vine (Vitis Vinifera), whenever produced by this method. The red vine leaves should preferably be picked in autumn at the time when they have the maximum red colouration.

The leaves should be dried away from the sun and in an atmosphere the temperature of which does not exceed 50° C. They may also be stabilised by conventional methods and in particular by the PERROT-GORIS process, i.e. by treatment with ethanol vapour in an autoclave to kill the ferments present in the fresh leaves.

The dry leaves, whether stabilised or not, are crushed so as to obtain a coarse powder (No. 60 Sieve). A greater fineness would not have any particular advantage.

This powder is then extracted by aqueous alcohol of such a nature that neither the

[*Price* 4*s.* 6*d.*]

pectins nor the cellulose nor the gums are extracted ; an ethanol content of 50% to 80% is suitable.

This operation should be carried out under heat in order to destroy the oxidases and other ferments and to coagulate the useless proteins. A temperature of 60 to 90° C is suitable.

This heating operation should take place within a relatively short space of time, preferably less than 1 hour, in order to prevent the activity from being destroyed, a destruction which could result from any prolongation of the heating time. A second stage in the extraction is the addition of a mineral or organic acid such as sulphuric, hydrochloric or acetic acids at concentrations of 1 to 5 parts per 1000 by volume of the volume of the solvent used. This addition of acid is absolutely necessary to conserve the natural red colouring of the leaf. It should only be carried out when the temperature has dropped to 40 to 50° C. When the mixture is acidified the extraction is carried out under agitation at the temperature of 40 to 50° C for several hours, but preferably between 1 to 5 hours. This extraction with aqueous alcohol, and subsequent acidification, may be repeated several time until colourless filtrates are obtained and the vegetable matter is exhausted.

The acidified filtrates, separated from the marcs, are distilled in a high vacuum, i.e. of the order of 5—10 mm. Hg., at a temperature varying between 20 and 50° C. The addition of an antioxidant is then indispensable and this antioxidant is preferably one authorised by medical authorities, e.g. ascorbic acid or a metabisulphite. The concentration of the antioxidant added is proportional to the amount of dry extrat which will be obtained from the filtrate as determined by measuring the amount of dry extract obtained from a sample of filtrate, and should be between 1 to 5 parts per 100 by weight of the latter. The evaporation of the solvent should be carried out until a dark red soft paste is obtained.

This paste must be freed of its impurities such as chlorophyll, waxes and fatty matter. To this end it may be extracted with a suitable water-immiscible non-polar solvent, such as benzene or carbon tetrachloride, or it may be directly dissolved in water, in which these impurities are insoluble. Dissolution in water for this purpose should be carried out at a temperature between 60 and 95° C., with agitation. The weight of water should correspond to one or two times the weight of the original plants. This water should be acidified, the concentration of the acid varying between 0.5 and 2 parts per 1000 by volume. It should contain an antioxidant of the same type as the first and shoyld be at a concentration of 1 to 5 parts per 1000 by weight. The liquid obtained is separated from solid matter by filtration or centrifuging.

There are then two possible ways for treating this liquid :—

1. If it is intended to obtain a dry product intended to serve as a raw material for the preparation of non-injectable pharmaceutical substances, the solution may either be concentrated under a vacuum of 15 to 20 mm. at a temperature between 40° and 50° C. until a dry powder is obtained, or the solution may be passed into a spray-drier (" extract 1 ").

2. For injectable preparations, the aqueous solution should undergo additional processes of purification intended to drive off substances which, in time, have a tendency to separate out and be precipitated. Thus, the solution is freed from tannins by saturation with sodium chloride. The profuse precipitate is separated by centrifuging or filtration, and the saline solution is then extracted with a solvent which is preferably an alcohol which will not mix with water and which can be an aliphatic monohydric alcohol having 4 or 5 carbon atoms in the molecule, in a counter-current extraction apparatus, or treated by any other similar process. This solvent should contain a proportion of mineral or organic acid varying between 5 and 10 parts per 1000 parts by volume. After complete extraction the alcoholic solution is concentrated to one tenth of its initial volume so as to precipitate part of the sodium chloride which is separated by filtration. This alcoholic solution is then dehydrated by passing it over a drying substance such as anhydrous sodium sulphate or calcium chloride.

In order to obtain a dry preparation comprising the active ingredients it is necessary either to desiccate the alcoholic solution in vacuo or to treat it by a solvent such as ether, petroleum ether or benzene, which precipitates a red powder (" extract 2 "). In both cases the powder is dried over P_2O_5 and stored away from light and damp. It is thus possible to obtain, from the red vine leaf, purified substances which it has been possible to analyse and on which it has been possible to prove that they have an activity of the same type as the natural product. Three aspects have been taken into account for this purpose :—

1. It has been necessary to make sure that the extracts 1 and 2 contain the same substances as the natural product and to identify these substances as far as possible.

2. It has been necessary to make sure that the extracts 1 and 2 had a similar pharmaceutical action to that of the leaf.

3. Finally it has been necessary to make sure that these extracts possess valuable properties for human treatment and that they were neither toxic nor were the cause of secondary reactions.

For the first test a solution obtained by the infusion of red vine (this infusion being the form of which proof of the therapeutic action was first demonstrated was compared with extracts 1 and 2 above. It is important to note that the infusion of red vine gives a red-brown solution, the colour of which passes to brilliant red upon slight acidification. This colouring passes into butanol. This peculiarity has been put to use and the following methods have been used :—

Chromatography on paper and ultra-violet and visible spectrum spectrophotometry.

The techniques used in particular were those of HARBORNE, see :—

The Chromatographic identification of anthocyanin Pigments ; Journal of Chromatography 1958, 1, 473.

The Chromatography of the flavonoid pigments—ibid. 1959, 2, 581.

Spectral methods of Characterising anthocyanins. The Biochemical Journal 1958, 70, 22.

If the rough infusion of red vine leaves is passed over Whatmann No. 1 paper with Partridge's solvent, (butanol : acetic acid : water in the proportions 4 : 1 : 5 by volume) the presence of anthocyanic pigments, flavones, tannin, catechin, sugars and esculoside will be noted.

In order to increase the accuracy of the method the following procedure was adopted :—

10 grams of red vine leaf were infused in 100 ml. of water. After cooling, the solution was filtered, saturated with sodium chloride and then extracted with 5 times 10 ml. of 1% hydrochloric butanol (Solution T).

1 gram of extract 1 was put in suspension in 100 ml. of hot water and subjected to the same treatment as the leaf : it was then extracted by 5 times 10 ml. of 1% hydrochloric butanol (Solution A).

0.2 gram of extract No. 2 was dissolved in 50 ml. of 1% hydrochloric butanol (Solution B).

The three solutions, T, A and B were then subjected to the same treatment ; to 10 ml. of each solution were added 40 ml. of diethyl ether. The resulting solutions (S) were each agitated 5 times with 5 ml. of 0.2% hydrochloric acid solution, the aqueous extracts of each of T, A and B were combined and the ether removed, one portion was hydrolysed by 20% HCl for 45 minutes and then extracted with isoamyl alcohol, and the other non-hydrolysed portion was extracted with butanol. Of these two latter solutions the first contains the anthocyani dines (aglucones) and the second the anthocyanosides (glucosides).

The residual solutions from the solutions (S) were then each agitated with 5 times 10 ml. of 2% carbonated water and the extracts obtained, freed from ether, were neutralised and then treated in the same way as the acid extracts.

The flavones (aglucones) and the flavonosides were thus made available separately. Each solution was then subjected to chromatographic and spectral analysis according to Harborne's or Venkataraman's processes (Progress in the chemistry of natural organic substances by Zechmeister 17th Volume, page 2, 1959 Spreinger-Verlag, Vienna). The results obtained principally allowed the monoglucosides of delphinodol and malvidol, of quercetine and quercetroside, of koempferol and traces of esculoside to be identified. These results were common to the leaf and to extracts 1 and 2. Therefore, the first object of the invention, i.e. to obtain an extract of red vine corresponding to the natural product, was thus achieved.

By this method it is possible to determine the content of active ingredients by a spectrophotometric measurement in the anthocyanidine solutions in the isoamyl alcohol on the one hand and in the solution of flavones on the other hand. The absorption spectrum of the anthocyanidine solution is found to be between 600 mμ and 400 mμ. A characteristic curve is obtained with a maximum of 540 mμ. Taking for the value $E1\%_{1\ cm.}$ that of the malvidine which is 1000, a concentration of 0.05 to 0.02% in the plant is arrived at, 0.25 to 1% in the product 1 (rough) and 1.4 to 5% in the purified product 2. Malvidine represents aglucone in the major portion of the anthocyanes present in the plant and it exists in the form of monoglucoside principally, according to chromatographic analysis.

The absorption spectrum of the solution of flavones is found to be between 400 mμ and 230 mμ. The characteristic curve has two maxima, one at 367—370 mμ and the other at 255 mμ. By taking the value $E1\%_{1\ cm.} = 630$ of quercetine, aglucone of the major portion of the flavonosides present in the plant, 3 to 6% in product (1) and 15 to 30% in the purified product (2). The differences in content generally stem from variations in the primary materials.

The accompanying drawings represent the spectrophotometric curves corresponding to the above double analysis and in which :—

Figure 1 shows the absorption spectrum of the solution of anthocyanine ; and

Figure 2 shows the absorption spectrum of the solution of flavone.

To summarise, the product according to the invention is defined by a high content of anthocyanosides and flavonosides, a content which is determined by the analytical technique described above.

On the other hand, this analytical technique has allowed it to be determined that

the products 1 and 2 thus obtained are perfectly stable in the course of time, and that in 6 months, for example, their anthocyane and flavone content has not varied more than 5 per 100 at most.

The same is true for the pharmaceutical forms and in particular the liquid forms, the content of active agents of which has not altered as much from the point of view of chemical determination as from the measurement of physiological activity such as the spasmolytic activity.

The second control operation is that of ensuring that the pharmaceutical actions of the leaf are maintained in the course of extraction and are present in extracts 1 and 2. This activity may be proved by conventional tests as follows :—

The antihyaluronidasic action on mice.

The action on the capillary resistance of guinea pigs which are lacking in a vitamin.

The spasmolytic action on the contraction of the isolated organ.

A. *Antihyaluronidasic action on mice.*

A. *Experimental procedure.*

(a) *Intravenously* (*Extract No.* 2).

Injection, 30 minutes before the test, of a given quantity of the extract dissolved in 0.9% sodium chloride.

Injection into the subcutaneous tissue of the skin of the abdomen of the mouse.

On the left hand side : 0.10 ml. of 1% Indian ink in 0.9% sodium chloride; on the right hand side : 0.10 ml. of 1% Indian ink in 0.9% sodium chloride at 9 per 1000. + 1, 2, 3 or 4 IU of hyaluronidase at 150IU/mg.

(b) *Orally* (*Extract No.* 1).

Administration, to mice, for three consecutive days, by probang, of the solution of Extract No. 1 or of the infusion of the red vine leaf.

The third day, 1 hour 45 minutes after the last administration, injection as above of Indian ink and hyaluronidase.

(c) *Reading of the results.*

Forty five minutes after the subcutaneous injections, the animals are killed and, after having slit the skin of the abdomen at its midportion, the skin is folded back over a plate of cork and the stains on the right hand and left hand sides are compared.

In the absence of an inhibitor the hyaluronidase brings about a diffusion of the Indian ink : the left hand stain is less extensive than the right hand stain.

(B). *Results.*

The following table shows the results of these experiments. The + sign indicates an anti-hyaluronidasic activity, the − sign indicates the absence of activity : the ± sign indicates a result which can be interpreted neither one way nor the other.

The experiment was carried out on about 350 mice.

Hyaluronidase in IU/Kg.	Extract No. 1 Via the bone		Extract No. 2 I. V.		Vine Leaf (Filtered Infusion.)	
	10 mg/Kg.	25 mg/Kg.	5 mg/Kg.	10 mg/Kg.	100 mg/Kg.	250 mg/Kg.
1	+	+	+	+	+	+
2	+	+	+	+	±	+
3	±	+	±	+	−	+
4	−	+	−	±	−	±

B. *Action on the capillary resistance of guinea pigs deficient in a vitamin.*

A. *Experimental procedure.*

A deficiency in vitamin C was obtained by the BURR and BURR diet with proportions of vitamins constituted by the vitamins A, D, E. B. B2, B6 and 10 mg/kg. of vitamin C. In theory only the deficiency factor C2 is lacking and in spite of the vitamin C content the diet tends to produce scurvy.

The experiments were carried out on 100 guinea pigs, the capillary resistance of which, measured with Professor PARROT'S praximeter (an apparatus measuring capillary resistance by the appearance of red patches on the skin), was, at the beginning, and in all cases, equal or greater than 30 cm. of mercury.

When they were considered sufficiently deficient in vitamins, the animals had a capillary resistance at the end of the experiment equal to or less than 10 cm. mercury. The capillary resistance was taken on the skin at the level of the gluteal muscle.

After the breaking down of the capillary resistance at the end of the vitamin deficiency, the products to be tested were administered

intraperitonally. Varied doses of Extract No. 2 were injected for three days. On the third day the capillary resistance was measured 2 hours after the last injection.

(B). *Results.*

These are collected in the table below. For each dose the results are given for a group of 5 to 10 guinea pigs.

Capillary resistance in cm. of Mercury	Red vine extract No. 2 injectable solution, intraperitoneal			Red vine leaf, solution obtained after filtration	
	10 mg/Kg.	20 mg/Kg.	50 mg/Kg.	250 mg/Kg.	500 mg/Kg.
10					
15					
20	↓				
25		↓		↓	
30					↓
35			↓		
40					
45 and above					

C. *Spasmolytic action on the isolated ileum of a guinea pig.*

A. *Experimental procedure.*

The musculotropic spasmolytic activity was measured on the ileum of a male guinea pig, which had been starved for 48 hours, according to the GADDUM technique (J. Exp. Physiol. 40(1) 49—74 (1955)) using a 5 ml. tank. The spasmolytic activity was evaluated in relation to the papaverine hydrochlorate with regard to the contractions brought about by doses of 10 and 100 grams of barium chloride according to the sensitivity of the organ.

B. *Results.*

	Quantity equivalent to the activity of 1γ of papaverine
Vine leaf (in infusion)	0.5 to 1 mg.
Extract No. 1	60 to 100
Extract No. 2	10 to 20 γ

Thus the physiological experiments allowed the value of the red vine and its extracts to be demonstrated.

EXAMPLE 1.

500 grams of leaves, picked as indicated above, were introduced into a laboratory apparatus of the Grignard type. 3.5 litres of aqueous ethanol containing 70% ethanol were added and, under constant agitation, it was boiled under reflux for 45 minutes. It was cooled to 40—50° C. and 25 ml. of pure hydrochloric acid added. Agitation and heating to 40—50° C. lasted for 2 hours. The filtrate was extracted and to it were added 2 grams of ascorbic acid.

2 litres of aqueous ethanol containing 70% ethanol were added once more to the marcs and extraction was once more carried out under agitation for 2 hours at 40—50° C. The new filtrate was extracted and added to the first and they were evaporated in vacuo at 40° C.

The soft extract obtained was dissolved under agitation by 500 ml. of 0.1% hydrochloric acid heated to about 90° C. The suspension was centrifuged and the solution concentrated in vacuo to 30% of its volume and then spray-dried (Extract No. 2).

In order to purify the product and to render it injectable, the solution was saturated before spray-drying with sodium chloride. The precipitate of tannins was eliminated by centrifuging and the aqueous solution was extracted in a counter-current apparatus with *n*-butanol containing 1% hydrochloric acid. The sodium chloride was separated by filtration and then the acid butanol solution was dehydrated by passing it over a layer of anhydrous sodium sulphate. The acid butanol solution was concentrated at a low temperature to 1/10 of its original volume. Finally 10 volumes of petroleum ether were added to the butanol solution. All the pigments were precipitated in the form of a dark red crystalline powder (Extract No. 2).

These two extracts served as raw materials for the preparation of pharmaceutical substances in the following way :—

EXAMPLE 2.

50 grams of extract were dissolved by agitation with 250 ml. of a mixture of ethyl alcohol, propylene glycol and water. The solution was filtered and conserved in a well-stoppered flask. 1 ml. of this solution is equivalent to about 2 grams of dry plant. This solution may be used for buccal administration.

EXAMPLE 3.

100 grams of extract were mixed with the following powder.

Amidon	10 grams.
Lactose	50 grams.
Kaolin	20 grams.
Levilite	10 grams.
Magnesium stearate	10 grams.

This was granulated when dry, coating was added to 0.4 gram and a pill was obtained containing 0.1 gram of red vine atomisate.

EXAMPLE 4.

100 grams of extract were moistened with sugar syrup and then granulated with enough sugar to form a granulated sugary preparation with 10 per 100 of red vine atomisate.

EXAMPLE 5.

100 grams of extract were mixed with 2.9 kilograms of an excipient for suppositories. The excipient can be either a eutectic mixture of fatty acid or glyceric esters of trilauric acid The suppositories contained 0.2 grams of red vine extract.

EXAMPLE 6.

The following substances were heated to 80° :—

Tween (Registered Trade Mark) 20	60 grams.
Span (Registered Trade Mark) 60	100 grams.
Stearic acid	125 grams.

and there were then added :—

Nipagin (Registered Trade Mark)	2 grams.
Nipasol (Registered Trade Mark)	3 grams.
Water	500 grams.

These substances were well mixed and left to cool to 35 to 40°. 20 grams of extract of red vine put suspended in water were added, and the whole incorporated by agitation. This was then made up to 1000 grams with water and homogenised. 100 grams of ointment contained 2 grams of extract. The ointment may also contain medicating ingredients such as derivatives of heparin, hydrocortisone or the extract of Hirudo medicinalis.

EXAMPLE 7.

Extract 2 was dissolved in a tampon solution of *p*H varying between 6.5 and 7.5 so that 1 ml. of that dissolved corresponds to 3 grams of plant. The solution was sterilised by filtration and antiseptically decanted into ampoules.

WHAT I CLAIM IS :—

1. A method for obtaining a substantially stable concentrated extract of red vine (Vitis Vinifera), in which dried and crushed leaves of red vine are extracted by warm aqueous alcohol, and the mixture is acidified after cooling, the filtered liquid then being distilled in vacuo in the presence of an antioxidant.

2. A method as claimed in Claim 1, in which the aqueous alcohol has an alcoholic content of 50 to 80% and the temperature of extraction is from 60 to 90° C.

3. A method as claimed in Claim 1 or 2, in which the acid is used in the ratio of 1 to 5 parts per 1000 by volume of the volume of the solvent at 40 to 50° C. for 1 to 5 hours.

4. A method as claimed in Claim 1, 2 or 3, in which the distillation in vacuo is carried out at 20 to 50° C., the antioxidant being used in the ratio of 1 to 5% by weight of dry extract.

5. A method as claimed in any one of the preceding claims, in which the product is further purified by extraction with an aliphatic monohydric alcohol having 4 or 5 carbon atoms in the molecule and then concentrated to the dry state, or put into injectable form.

6. Methods according to Claim 1 for obtaining substantially stable concentrated extracts of red vine (Vitis Vinifera) substantially as hereinbefore described with reference to the specific Example 1.

7. Substantially stable concentrated extracts of red vine (Vitis Vinifera) whenever produced by a method as claimed in any one of the preceding claims.

8. Substantially stable concentrated extracts of red vine (Vitis Vinifera) substantially as hereinbefore described with reference to the specific Example 1.

9. Pharmaceutical compositions containing substantially stable concentrated extracts of red vine (Vitis Vinifera) as claimed in Claim 7 or 8.

10. Pharmaceutical compositions substantially as hereinbefore described with reference to the specific Examples 2 to 7.

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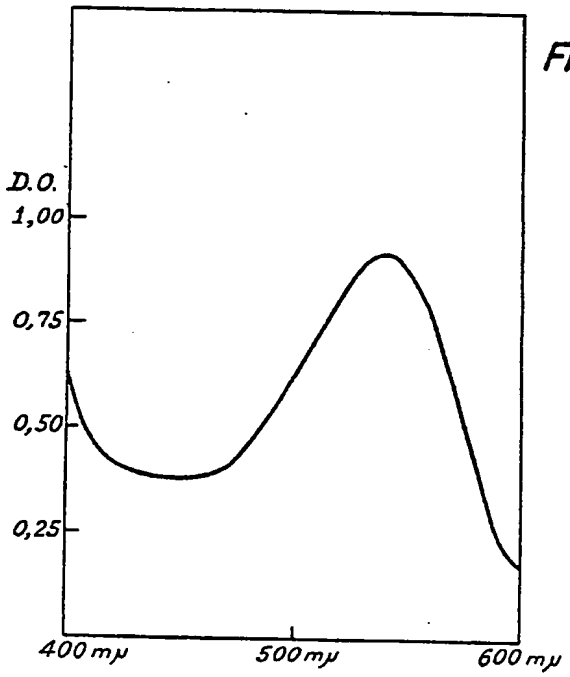

Fig. 1

Fig

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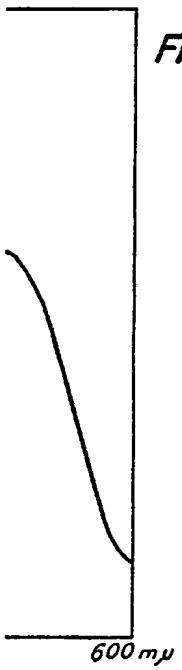

Fig. 1

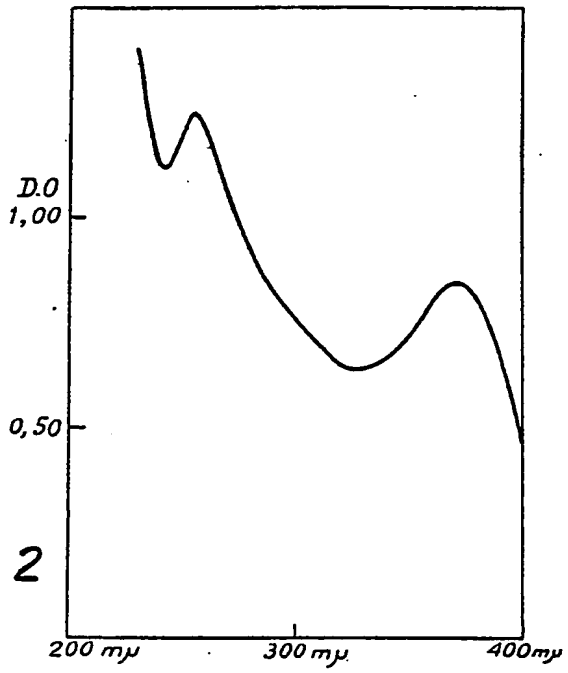

Fig. 2

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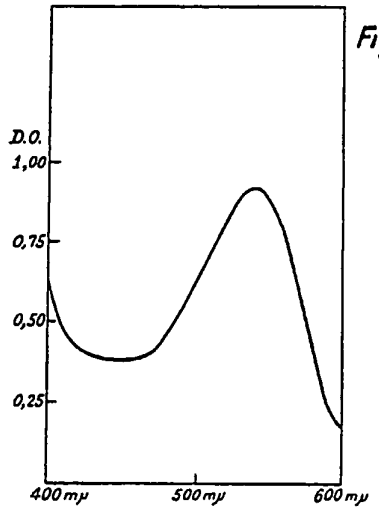

Fig. 1

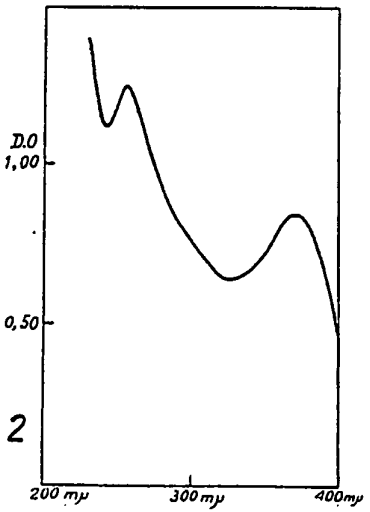

Fig. 2